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INOCULATION EFFECTS ON LEGUMES GROWN IN SOIL PREVIOUSLY TREATED WITH SEWAGE SLUDGE

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Summary—The effects on legumes of the long-term land application of sewage sludge are not clear. Sludge-borne toxic elements, as well as essential nutrients and organic matter, complicate the response of legumes in association with their symbionts. To examine this problem, several strains and species of *Rhizobium* and *Bradyrhizobium* were studied for their response to the presence of heavy metals in agar growth media. *Bradyrhizobium japonicum* was by far the most metal-tolerant of the organisms examined, often able to tolerate several times more Zn and Cd in agar media than more metal-sensitive organisms, such as *Rhizobium leguminosarum* bv. *trifolii*. Soils were subsequently collected from metal-contaminated plots located at the Woburn Market Garden Experiment, and rhizobia were inoculated into these soils and sown with the appropriate homologous legume. Uninoculated controls and unamended soils were also sown. White clover (*Trifolium repens*) in the uninoculated, sludge-amended treatment contained numerous, small white and ineffective nodules. Inoculation enhanced nodulation and plant growth, but not to levels observed for plants grown on soil not amended with sludge. Inoculation with effective strains of rhizobia had little effect on plants grown in control soils since an indigenous, effective population compatible with white clover was present in adequate numbers. Only soybeans (*Glycine max*) responded to inoculation since soybeans had never been cultivated in these soils. These results confirm earlier observations that *R. leguminosarum* bv. *trifolii* in soils exposed to sludge-borne metals for many years are ineffective. Microbial species, chemical characteristics of the soil into which the sludge was added and the length of time the microbes were exposed to the metals each affected the response of the macro- and microsymbionts.

INTRODUCTION

There is conflicting evidence on the effects of sewage sludge on microorganisms. Uncertainty arises from the fact that effects are specifically related to the composition of the sludge, soil microbial populations, chemical properties of the recipient soil, and the length of time that the microorganisms are exposed in soil to the sludge and sludge-borne metals. Generalizations regarding sludge effects on soil microorganisms, especially rhizobia, are difficult because so many factors affect the responses. However, the economic importance of rhizobia and their symbiotic relationship with legumes necessitates their study in sludge-amended soil.

Sewage sludge contains a variety of materials that are potentially toxic to rhizobia, including soluble salts, heavy metals and synthetic organics. Sludge-born salts have been shown by Madariaga and Angle (1992) to be toxic to rhizobia, as rhizobia are relatively salt-sensitive microorganisms (Singleton *et al.*, 1982). Numerous studies have shown that heavy metals may be toxic to rhizobia when present in soil in moderate to high concentrations (Reddy *et al.*, 1983: McGrath *et al.*, 1988; Giller *et al.*, 1989; McGrath, 1993) and may reduce the rate of symbiotic N_2-fixation (McGrath *et al.*, 1988). Hence, when a susceptible microbial population is exposed to sludge-borne toxic materials, adverse effects on that population may develop.

Sludge, however, also contains numerous components required for microbial growth. Additions of sludge-borne nutrients, micronutrients and organic matter to soil (Bouldin *et al.*, 1985) may enhance the growth of rhizobia in sludge-amended soil (Madariaga and Angle, 1992). The addition of sludge to soil also improves its physical properties (Epstein *et al.*, 1976), that in turn, may enhance the survival and growth of rhizobia. Coppola (1983), Speaker and Sopper (1988) and Pichtel and Hayes (1990) have shown that the addition of sewage sludge to soil, especially soil low in organic matter such as mine spoils, significantly increases the population and activity of soil microorganisms. Kinkle *et al.* (1987) and Heckman *et al.* (1987a, b) have demonstrated that the soil population of *Bradyrhizobium japonicum*, the microsymbiont of soybeans, increased during a 10 yr period after the application of high rates of

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metal-contaminated sludge. They suggested that heavy metals and other toxic components of sludge were not present in soil in concentrations sufficient to affect adversely *B. japonicum*. Martensson and Witter (1990) have reported that a significant number of *Rhizobium leguminosarum* bv. *trifolii* were present in soils amended 30 yr ago with sewage sludge, although the isolates displayed a delay in nodulation.

Contradictory observations have been reported in a number of other studies. McGrath *et al.* (1988) and Giller *et al.* (1989) have shown that only ineffective isolates of *R. leguminosarum* bv. *trifolii* survived in soil amended with metal-contaminated sludge for 30–50 yr. They noted that antagonistic effects were related to sludge-borne metals, notably Cd, Zn and Cu. Reddy *et al.* (1983) reported that the population of *B. japonicum* USDA 110 was reduced when exposed in soil amended with high rates of sewage sludge. Heavy metals were suggested as the cause of the decline, although sludge-borne soluble salts are a more likely explanation for the short-term effects described (Madariaga and Angle, 1992).

Our objective was to determine why the effects of sludge and sludge-borne metals on rhizobia are not consistent. Understanding the nature of the cause and effect relationship between sludge and rhizobia may reduce problems related to sludge application to land cultivated to legumes.

MATERIALS AND METHODS

Determination of heavy metal toxicity to rhizobia

Isolates of both slow- (*Bradyrhizobium*) and fast-growing (*Rhizobium*) rhizobia were obtained from a variety of sources. Isolates of *R. leguminosarum* bv. *trifolii* were obtained from nodules grown on plots that had received sludge (S-isolates) or farm yard manure (FYM) (F-isolates), as described by Giller *et al.* (1989). USDA strains of diverse geographical origin were obtained from Peter van Berkum, USDA-ARS, Beltsville, Md. Additional isolates of *R. meliloti*, *B. japonicum*, *R. leguminosarum* bv. *phaseoli*, *R. fredii*, and *R. leguminosarum* bv. *viciae* were obtained from the Rothamsted *Rhizobium* Culture Collection (now held by M. Dye, Institute for Grassland and Animal Production, Welsh Plant Breeding Station, Plas Gogerddan, Dyfed, Wales).

All isolates were grown in HEPES–MES (HM) [N-2-hydroxyethpiperazine-N-2-ethanesulfonic acid; 2-(N-morpholino) ethanesulfonic acid] minimal broth medium with arabinose (0.1%) to mid-log phase (Cole and Elkan, 1973). Cultures were maintained on HM medium slants at 4°C. Before use, cultures were centrifuged, the supernatant discarded, the cells resuspended in an equal volume of distilled water, and the washing procedure repeated three times.

Agar plates of HM medium supplemented with individual concentrations of Zn, Cu, Cd and Ni were prepared. Zinc was added as $ZnSO_4 \cdot 7H_2O$ at concentrations from 10 to 500 μg Zn ml^{-1}. Copper was added as $CuSO_4 \cdot 5H_2O$ from 0.25 to 10 μg Cu ml^{-1}; Cd was added as $CdSO_4 \cdot 8H_2O$ from 0.5 to 10 μg Cd ml^{-1}; and Ni was added as $NiSO_4 \cdot 7H_2O$ from 0.5 to 20 μg Ni ml^{-1}. At least eight concentrations were used for each metal. Washed cells of each rhizobial suspension were streaked onto the surface of the metal-amended agar, the plates were kept at 28°C for 6 days (10 days for *B. japonicum*), inspected for growth, and the maximum resistance level (MRL) determined. The MRL is defined as the highest metal concentration that permits visible growth.

Soil and inoculation effects on nodulation and plant growth

Soils of the Cottenham series were obtained (0–15 cm deep) from the Market Garden Experiment at Woburn (England) to which sewage sludge or FYM had been added from 1942 to 1961. An unamended control soil was also obtained from the same area. Metal speciation in the sludge-amended plots was stable, as a result of the long equilibration period. Except for metal content, most other soil characteristics and variables were identical among the soils (McGrath, 1984, 1987). After collection, soils were maintained moist at 4°C.

2 kg of soil were added to pots (20 cm) that were maintained in a greenhouse. 10 ml of a log phase culture of each of the rhizobial strains was poured onto a 4 cm dia area of surface soil in the centre of each pot. Pots were then sown, as appropriate for the inoculum, with one of the following legumes: soybean (*Glycine max* L. Merr. cv. Lee), French bean (*Phaseolus vulgaris* L.), White clover (*Trifolium repens* L. cv. Blanca), Crimson clover (*T. incarnatum* L.), and Alsike clover (*T. hybridum*). Seedlings were thinned to one plant per pot for the large-seeded legumes (soybeans, beans) and to ten plants per pot for the small-seeded legumes (clovers). Plants were grown for 8 weeks. Pots were watered with distilled water as needed.

At harvest, the shoots and roots were separated. Shoots were dried at 70°C for 24 h, weighed, ground to pass through a 2 mm pore-size stainless steel sieve and after semimicro Kjeldahl digestion, the total N content determined by an automated indophenol blue method (Varley, 1966). The soil was gently washed from the roots. Nodulation was assessed by estimating the number of nodules per plant, noting nodule location on the roots and the size and color (as an index of the potential for nitrogen fixation) inside the nodules. Roots were then dried as above and weighed.

Statistics

MRL values were determined in two separate, but identical, experiments. In each experiment, the MRL values were identical. When rhizobia were inoculated into soils, each soil–plant combination was replicated three times. Pots were randomized within a soil treatment, but inoculation treatments were not randomized, to avoid cross contamination. Treatment means were separated, at the 5% level of probability, using an LSD test (SAS, 1985). Inoculation means were combined, as the variances for each treatment were not statistically different.

RESULTS

In vitro assay of metal sensitivity

Nickel and Cu were the most toxic of the metals examined, with MRL values of about 1–2 μg metal ml^{-1}, whereas Cd exhibited slightly less toxicity (Table 1). Zinc was much less toxic than any of the other metals examined, occasionally being 100-fold less toxic than Cu or Ni. Metal toxicity rankings were generally in the sequence reported by Kinkle *et al.* (1987) for rhizobia, with only Cd being more toxic in our study.

Differences in metal sensitivity were detected between the various isolates and species. The S-isolates were more resistant to the toxic effects of Zn than the F-isolates from plots with a lower metal content. S-isolates were able to tolerate *ca* 4.5 times the amount of Zn in the growth medium than the F-isolates. An increase in Cu tolerance was also observed for the S-isolates in comparison to the USDA strains and F-isolates. The S-isolates were approximately two times more tolerant to Cu than the USDA strains and four times more-tolerant than *R. leguminosarum* bv. *viciae*, *R. fredii* and *R. meliloti*. Although the Woburn sludge-amended soils were also contaminated with Cd and Ni, the selection pressure was apparently not adequate to elicit a response. Chaudri *et al.* (1992) were able to detect a distinct Cd response between the same S- and F-isolates. Apparently the use of metal-amended agar is a less sensitive assay method than the use of solutions as employed by Chaudri *et al.* (1992).

Table 1. Maximum resistance level (MRL) of various strains and species of *Rhizobium* and *Bradyrhizobium* to heavy metals

Strains or species	MRL (μg ml^{-1})			
	Zn	Cu	Cd	Ni
R. leguminosarum bv. *trifolii* S1*	100	1.0	2.0	2
R. leguminosarum bv. *trifolii* S2	100	1.5	2.0	2
R. leguminosarum bv. *trifolii* S3	200	2.0	2.0	2
R. leguminosarum bv. *trifolii* F3**	30	1.0	2.0	2
R. leguminosarum bv. *trifolii* F6	50	0.75	2.0	2
R. leguminosarum bv. *trifolii* F7	30	0.75	2.0	2
R. leguminosarum bv. *trifolii* USDA 2230	100	0.50	2.0	1
R. leguminosarum bv. *trifolii* USDA 2235	100	0.75	2.0	1
R. leguminosarum bv. *trifolii* USDA 2227	100	0.75	2.0	2
R. leguminosarum bv. *trifolii* USDA 2238	100	0.75	2.0	2
R. leguminosarum bv. *trifolii* USDA 2237	100	0.75	2.0	2
R. leguminosarum bv. *trifolii* USDA 2234	100	0.75	2.5	2
R. leguminosarum bv. *phaseoli*	75	0.50	2.0	2
R. leguminosarum bv. *viciae*	20	0.50	2.0	2
R. meliloti	20	0.50	2.5	4
R. fredii	75	0.50	2.0	3
B. japonicum	500	5.0	7.5	5

*S = isolates from sludge-amended soil.
**F = isolates from FYM-amended soil.

The USDA strains of *R. leguminosarum* bv. *trifolii*, collected from throughout the world, were all more resistant to Zn than the F-isolates of the same species isolated in the U.K. This was not observed for any of the other metals. The U.K. isolates apparently represented a Zn-sensitive population of *R. leguminosarum* bv. *trifolii*, that may potentially be responsible for the metal toxicity observed in soils from the Woburn sludge-amended soils. Metal tolerance of the remaining species was variable and was dependent on the metal and the species. However, *B. japonicum* was the most tolerant species. For example, *B. japonicum* tolerated five times the amount of Zn in the growth medium than compared to most strains of *R. leguminosarum* bv. *trifolii*.

Pot study

Inoculation of soybeans with *B. japonicum* was successful, with many large nodules, located along the tap root, that were pink indicating active N_2-fixation. Inoculation was also successful for beans where plants grown in the control soil contained only a few nodules located along the lateral roots, whereas inoculation resulted in numerous, pink nodules scattered along the tap root.

Nodulation of clover was more closely related to the soil to which the inoculum was applied. In the uninoculated FYM soils, clover was nodulated with moderate numbers of large nodules that were pink within. In the sludge-amended soil, however, plants contained many extremely small, white nodules. McGrath *et al.* (1988) reported the same observation and indicated that these nodules were formed from ineffective rhizobia, all of relatively uniform genetic composition (Giller *et al.*, 1989). McGrath and Giller attributed the loss of the ability to fix N_2 to the selection of a metal-tolerant, ineffective strain by heavy metals in soil.

Inoculation of clover was only moderately successful in the sludge-amended soil and produced a few large nodules that were pink, although most nodules were small and white. This observation is, to some extent, contrary to that of Giller *et al.* (1989) who reported that inoculation resulted in amounts of nodulation and N_2-fixation equivalent to that found in the control soils. They, however, used a higher rate of inoculum and mixed it thoroughly into the soil which may have accounted for this difference. Inoculation of the FYM soils had no significant effect on nodulation, probably because the indigenous N_2-fixing population was adequate for nodulation, and inoculation into a stable population of indigenous rhizobia is seldom successful (Alexander, 1977).

Plant responses to soil and inoculation are presented in Table 2. Root weight was not significantly affected by inoculation, whereas the effect of soil in which the plants were grown was significant. For almost every combination of plant species and inoculum, root weights were lowest in the sludge-amended soil. The greatest reduction in root weight was

Table 2. Soil and inoculation effects on legume growth in farm yard manure (FYM)-, control- and sludge-amended soils

Soil	Species	Root weight (g)		Dry shoot weight (g)		N (%)		Total shoot N (mg)	
		Inoc. +	Inoc. −	Inoc. +	Inoc. −	Inoc. +	Inoc. −	Inoc. +	Inoc. −
Sludge	Crimson clover	0.15	0.14	0.41	0.41	1.68	1.45	8.31	6.1
	Alsike clover	0.29	0.21	0.71	0.59	2.65	2.79	19.7	16.5
	White clover	0.24	0.18	0.62	0.50	2.57	2.00	20.0	10.9
	Soybean	0.35	0.41	1.26	1.30	3.21	1.37	43.3	18.0
	Bean	0.21	0.22	1.11	0.63	2.83	1.91	34.7	11.5
	$\bar{X}$	0.25	0.23	0.82	0.69	2.59	1.90	26.9	12.6
FYM	Crimson clover	0.40	0.43	1.73	1.46	2.89	2.69	53.6	61.9
	Alsike clover	0.30	0.32	1.16	0.98	3.39	3.43	29.6	43.6
	White clover	0.23	0.36	0.94	1.22	3.35	3.23	41.0	46.2
	Soybean	0.58	0.66	2.66	1.82	3.33	1.40	93.6	29.9
	Bean	0.32	0.40	2.54	1.98	2.43	2.31	73.4	44.7
	$\bar{X}$	0.37	0.43	1.81	1.49	3.08	2.62	58.2	45.3
Control	Crimson clover	0.39	0.44	1.85	2.32	2.87	2.98	49.9	44.6
	Alsike clover	0.22	0.31	0.87	1.28	3.20	3.21	37.3	31.6
	White clover	0.32	0.28	1.22	1.42	3.30	3.18	30.6	39.1
	Soybean	0.65	0.72	2.82	1.78	2.25	1.22	77.5	22.3
	Bean	0.35	0.38	3.06	1.29	2.67	2.93	67.8	62.5
	$\bar{X}$	0.39	0.43	1.96	1.62	2.86	2.70	52.6	40.0
$LSD_{(Soil \times species)}$		0.11		0.23		0.22		6.6	
$LSD_{(Soil)}$		0.14		0.30		0.28		8.6	

observed with crimson clover grown in the sludge-amended soil.

Shoot weights were significantly lower in the sludge-amended soils than in the control or FYM-treated soils (Table 2). The differences in shoot weight of plants grown in control and sludge-amended soil were several fold. However, no obvious symptoms of phytotoxicity were evident.

Inoculation did not significantly affect shoot weight in the sludge-amended soil. However, it significantly increased shoot weight for soybeans and beans in the control soils. For example, shoot weight of beans was increased > 2-fold by inoculation with an effective strain of rhizobia. Control soils contained low numbers of indigenous *R. leguminosarum* bv. *phaseoli* and, thus, the beans benefitted from inoculation. The population of *R. leguminosarum* bv. *trifolii* in the control soil was adequate to nodulate clovers and, thus, inoculation had no effect.

The total and percent N in the shoot were increased by inoculation with the appropriate N-fixing microsymbiont. The increase in the N content of the shoot, as the result of inoculation, was greatest in the sludge-amended soil where the soil rhizobial population was affected by the high metal content. The N content was extremely low in plants grown on uninoculated sludge-amended plots.

DISCUSSION

Our study largely resolves the apparent conflict between previous studies on the effects of sewage sludge on legume growth. The relevant question is why were the results reported by Kinkle *et al.* (1987) and Heckman *et al.* (1987a, b) on the one hand and those of McGrath *et al.* (1988) and Giller *et al.* (1989) on the other considerably different from one another? This question is extremely important in assessing the safety and potential utility of applying sewage sludge to land cultivated to legumes.

It is unlikely that either soluble salts or toxic organics were responsible for any of the differences, as salts are flushed from soil relatively quickly (Angle *et al.*, 1992), and toxic organics are chemically and biologically degraded over several years (Angle and Baudler, 1983). Several factors, however, may individually or in concert have resulted in the discrepancy between the various studies.

The most striking and obvious explanation for the difference between the U.K. and U.S.A. studies where sludge was added to soil is that the total metal content in the Woburn soils, U. K. was considerably higher than that of the Beltsville soils, U.S.A. (Table 3). The contents of all metals examined were approximately three to five times higher in the Woburn plots than in the Beltsville plots. Chaudri *et al.* (1992) reported that Cu is one of the most important elements in determining metal toxicity in soil. The Beltsville plots contained relatively low amounts of Cu compared to the most contaminated of the Woburn plots.

Another significant difference between the U.S.A. and U.K. studies was that the rhizobial species present were different. Borges and Wollum (1980, 1981) and Kinkle *et al.* (1987) have shown that there is a distinct difference in the metal sensitivities of fast-

Table 3. Cation-exchange capacity (CEC) and total metal content (Aqua regia digest) of U.S.A. (Beltsville) and U.K. (Woburn) sludge-amended plots that exhibited decreased white clover yields

Site	CEC ($cmol\ kg^{-1}$)	Element ($\mu g\ g^{-1}$ soil)			
		Zn	Cu	Ni	Cd
Beltsville*	10.4	119–151	31–35	11–23	1–5
Woburn†	12	180–435	70–150	22–33	6–13

*R. L. Chaney, unpublished data.
†S. P. McGrath (1993).

(*R. fredii*) and slow-growing (*B. japonicum*) rhizobia. The thick polysaccharide capsule around bacterial cells, especially around the slow-growing bradyrhizobia, binds and sequesters metals, thus preventing uptake into the cell (Beveridge and Doyle, 1989). Exclusion of Cd by the polysaccharide capsule has been demonstrated for *Klebsiella aerogenes* (Bitton and Freihofer, 1978) and *Escherichia coli* (Mitra *et al.*, 1975) as a means of protection. Slow-growing bradyrhizobia also cause an alkaline reaction within their immediate niche, thereby reducing metal solubility and activity of most metals (Alexander, 1977). Fast-growing rhizobia usually reduce the pH of their environment, thereby enhancing metal availability and toxicity. Within fast-growing species of rhizobia, there was also a range of metal sensitivities. *Rhizobium leguminosarum* bv. *trifolii* was the most sensitive of all the species examined. Differences of 2–6-fold were occasionally observed in the MRL of species. In addition, most of the isolates of *R. leguminosarum* bv. *trifolii* from the Woburn FYM plots were more metal-sensitive than isolates from the USDA culture collection, suggesting that the isolates from Woburn were intrinsically more metal-sensitive.

Another consideration that may explain the differences between the U.S.A. and U.K. results is the length of time that the rhizobia were exposed to the metals. Sewage sludge was applied to the Beltsville plots in 1975, and between 1942 and 1961 at the Woburn plots. Adverse effects of sludge on the population of rhizobia may require several decades to develop, and the Beltsville plots are of insufficient age for the adverse effects to become apparent.

On the basis of this and previous studies, we conclude that the application of sludge to land that may be cultivated to legumes requires caution. Sludge application has been shown to have adverse effects on white clover, although the conditions that resulted in these adverse effects are not fully understood. Inoculation can partially compensate for any sludge related reductions in nitrogen fixation and plant growth. Future studies are needed to better define under which conditions adverse effects occur.

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